## REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

## I. CLAIM STATUS & AMENDMENTS

Claims 22, 23, 27-33 and 47-84 were pending in this application when last examined.

Claims 66, 67 and 69-80 were examined on the merits and stand rejected.

Claims 22, 23, 27-33, 47-65, 68 and 81-84 were withdrawn as non-elected subject matter.

Support for the amendments to claims 66, 69, 70, 73, 77-79 and 82 can be found in the disclosure, for instance, in Example 6 on pages 98-102 and original claims 24-25, 34-35, 45, 47-48 and 58.

Claim 69 (element 2) is amended to change "a" to "the" before "reference library" and "reference sample" to recite the proper antecedent basis for the terminology.

New claim 85 has been added. Support for new claim 85 can be found in the disclosure, for example, in Figure 6 on page 83, lines 16-30, page 84, lines 1-2; Example 5 on pages 96 to 97 and page 98, lines 1-9.

Claims 71-79 are amended to change their dependency from claim 70 to claim 69.

No new matter has been added.

Claims 67-68, 70 and 80-81 have been cancelled without prejudice or disclaimer thereto. Applicants reserve the right to file a continuation or divisional application on any cancelled subject matter.

Claims 22, 23, 27-33, 47-66, 69, 71-79 and 82-85 are pending upon entry of this amendment.

## II. UNEXAMINED SUBJECT MATTER

At the bottom of page 2 of the Action, it was indicated that claims 66, 67 and 69-80

encompass unelected inventions drawn to detecting sequences (i.e., methods of detecting unelected sequences of SEQ ID NOS: 12-19) other than elected SEQ ID NO: 11. It was indicated that the Office has not examined these unelected sequences.

Applicants respectfully traverse this position and submit that this aspect of the claims should be examined with the elected invention. In other words, please examine elected SEQ ID NO: 11 and the combination of elected SEQ ID NO: 11 with other sequences for the reasons set forth below.

The present invention is a diagnostic method, which involves looking at SEQ ID NO: 11, but not only at this sequence alone. Rather, it involves looking at SEQ ID NO: 11 and a collection of other sequences. The invention is still directed to elected SEQ ID NO: 11 as this sequence is the minimum component of the collection of sequences to be analyzed. In this sense, the objected and rejected claims are not directed to SEQ ID NOs. 12 to 19, but to elected SEQ ID NO: 11, together with one or more other sequences (i.e. SEQ ID NOs. 12, 13, etc.).

Accordingly, the diagnostic method of the invention is based on SEQ ID NO: 11, but preferably not on SEQ ID NO: 11 alone as a single sequence, but on several different sequences, since using a collection of 2, 3 or more sequences gives a more reliable diagnostic result.

In view of the above, it is respectfully submitted that the claims should be examined with respect to elected SEQ ID NO: 11, and a combination of elected SEQ ID NO: 11 with other sequences as this is the gist of the invention.

On page 3 of the Action, it is indicated that claims 66, 67 and 69-80 encompass unelected species of ailments. In reply thereto, kindly note that the claims have been amended to the elected species of hepatocellular carcinoma as the species ailment. In particular, amended claim 66 now recites "a method of diagnosing hepatocellular carcinoma" and claims 69, 70, 77-79 and 82 have been amended to recite "hepatocellular carcinoma" instead of "liver disorders or epithelial cancer."

## III. CLAIM OBJECTION

On page 3, claim 73 was objected to on the basis that the recited methods therein are "detection methods" and not "comparison methods." See the middle of page 3.

The present amendment overcomes this objection. In particular, claim 73 is amended to recite "is detected by" instead of "is compared by."

Thus, the objection to claim 73 is untenable and should be withdrawn.

## IV. INDEFINITENESS REJECTION

Claims 66, 67 and 70-80 were newly rejected under 35 U.S.C. § 112, second paragraph, as indefinite for the reasons set forth on pages 3-5. The Office indicated that this was a new ground of rejection necessitated by amendment.

This rejection is respectfully traversed as applied to the amended claims.

Based on a definition of the term "identifying said nucleic acid(s) detected in step (a) which is differentially expressed in the sample isolated from the patient compared to the reference library or the reference sample" (page 66, lines 29–31 and page 67, lines 1-5), it is understood to mean selecting said nucleic acid(s) which is(are) differentially expressed compared to the reference library or the reference samples which fulfills the following criteria: the level of differential expression of the detected said nucleic acid(s) compared to the reference library or the reference samples is greater than about 2 fold, preferably greater than about 5 fold, more preferred greater than about 10 fold upregulated. See Figure 1 (RNA expression levels in HCC) wherein ObcI5 (SEQ ID NO:11) is upregulated greater than 2 fold when compared to RNA isolated from normal liver. In this regard, please see new claim 85.

Since claim 66 has been limited to identification of SEQ ID NO: 11, further data are related to the expression pattern of this nucleotide sequence only. Overall, DNA chip, RT-PCR and immunohistochemistry data show highly specific up-regulation of RNA of SEQ ID NO: 11

in hepatocellular carcinomas (HCCs) as compared to reference library/sample represented by RNA isolated from normal liver (non-neoplastic) or other neoplastic tissues. Namely, the mean SEQ ID NO: 11 RNA expression level exceeds all other normal and neoplastic tissues tested at least by factor 8. See, for example, Figures 1 and 2, and page 23, lines 20-21.

The quantitative RT-PCR results mirror the cDNA microarray data (page 23, lines 27-28). *In situ* hybridization reveals the specific and strong expression of SEQ ID NO: 11 RNA in cytoplasm of hepatocarcinoma cells, whereas it is not detected in tumor stroma and non-neoplastic liver cells (page 24, lines 6-9).

Furthermore, in the siRNA mediated knock down of SEQ ID NO: 11 expression in the hepatocarcinoma cells, it is determined that the level of mRNA encoding the tumor suppressor gene retinoblastoma protein 1 (RB1) is up-regulated several fold, in a dose dependent manner (Figure 6 on page 83, lines 16-30, page 84, lines 1-2; Example 5 on pages 96 to 97 and page 98, lines 1-9). This data supports the role of SEQ ID NO: 11 in detection (and treatment) of hepatocarcinoma, wherein the elevated expression of SEQ ID NO: 11 RNA may provide a negative regulation of the RB1, and therefore facilitate tumor cell growth.

Also, kindly note that new claim 85 further defines the level of positive upregulation (over-expression) of the identified polynucleotide(s) to a range "greater than about 2 fold" as supported by the above-identified disclosure.

Claim 70 has been cancelled, thereby obviating the lack of antecedent basis rejection for recitation of the term "the reference sample" in step 3 of claim 70.

To further clarify how polynucleotides are to be matched or what kind of match (steps (3 & 4) of claim 70) is indicative of disease of hepatocellular carcinoma, Applicants note the following.

Based on the definition of the term "matching said nucleic acid(s) identified in step (3) with said nucleic acid(s) differentially expressed in a pathologic reference sample or pathologic reference library" (page 67, lines 6-15), it is understood to mean that said nucleic acid(s)

identified in step (3) is (are) compared with said nucleic acid(s) differentially expressed in a pathologic reference sample or pathological reference library. Then said nucleic acid(s) identified in step (3) that is (are) also differentially expressed in the pathologic reference sample or pathologic reference library is (are) matched. In other words, said identical pair is identified and allocated. Since the differential expression of said nucleic acid(s) in the pathological reference sample or pathological reference library is (are) indicative of a disorder according to the invention (i.e. hepatocellular carcinoma (HCC)), such correspondence with the differential expression in the sample then indicates that the patient suffers from HCC (experimental data related to SEQ ID NO: 11 as discussed above).

In view of the above, it is respectfully submitted that the skilled artisan would clearly understand the metes and bounds of the "matching" step in element (4) of claim 70. Nonetheless, for the sole purpose of expediting prosecution and not to acquiesce to this rejection, claim 70 has been cancelled without prejudice or disclaimer thereto.

In view of the above, the 112, second paragraph, indefiniteness rejection of claims 66, 67 and 70-80 is untenable and should be withdrawn.

## V. ENABLEMENT REJECTION

On pages 5-12 of the Action, claims 66, 67 and 70-80 were newly rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification lacks enablement for a method of diagnosing every liver disorder, but is enabled for diagnosing hepatocellular carcinoma in patient comparing the level of expression of a polynucleotide comprising the sequence of SEQ ID NO: 11 in a first blood sample from said patient with the expression level of said polynucleotide in a second corresponding blood sample from a subject known to be free of hepatocellular carcinoma, i.e., detection of SEQ ID NO: 11 or a sequence comprising SEQ ID NO: 11 in a blood sample from HCC patient. The Office cited Horne et al. and Tockman et al. as evidence of the unpredictability in the art.

It is respectfully submitted that the present amendment overcomes this rejection for the claims have been amended to the subject matter indicated as enabled, i.e., detection of hepatocellular carcinoma by detecting SEQ ID NO: 11 or a sequence comprising SEQ ID NO: 11 in a blood sample from HCC patient. Again, support can be found in the disclosure, for instance, at page 66, lines 29–31, page 67, lines 1-5, and Example 6 on pages 98-102.

Further, attached herewith is Panzitt et al., Gastroenterology, Vol. 132, No. 1, pp. 330-342 (2007), which is further evidence that the instant disclosure is enabling for the claimed invention of detecting hepatocellular carcinoma. The authors of this paper include the Applicants. Panzitt et al. is related to characterization of the SEQ ID NO: 11 ("HULC") gene. The authors found that this novel gene is involved in striking up-regulation in hepatocellular carcinoma, as non-coding RNA. They found that this gene (i.e., HULC = the present invention) is the most up-regulated gene in HCC and was detected in blood of HCC patients. The authors concluded that the gene has use as a novel biomarker for HCC. See the abstract.

Therefore, the 112, first paragraph, enablement rejection of claims 66, 67 and 70-80 is untenable and should be withdrawn.

## VI. ANTICIPATION REJECTION

On pages 12-17, claims 66, 67 and 69-80 remain rejected under 35 U.S.C. § 102(b) as anticipated by Horne et al. (WO 02/29103), cited in the last Action. The Office was unpersuaded by the arguments in the last response.

This rejection is respectfully traversed as applied to the amended claims.

Based on the statements on page 17 of the Action, it appears the Office maintained the rejection on the basis that Applicants are arguing limitations not in the claims. For instance, on page 17, it was indicated that "the claims are not drawn to a specific diagnosis of hepatocyte carcinoma."

In reply thereto, please note the amended claims are drawn to a specific diagnosis of hepatocellular carcinoma. In particular, amended claim 66 calls for a method of diagnosing-hepatocellular carcinoma, said method comprising:

(1) identifying a polynucleotide in a sample from a patient, wherein said polynucleotide is a polynucleotide consisting of the polynucleotide sequence of SEQ ID No. 11 or a polynucleotide comprising the polynucleotide sequence of SEQ ID No. 11,

alone or in combination with at least one polynucleotide consisting of a polynucleotide sequence selected from SEQ ID Nos. 12-19 or a polynucleotide comprising the polynucleotide sequence selected from SEQ ID Nos. 12-19, and

(2) comparing expression of the polynucleotide(s) identified in step (1) with expression of said polynucleotide from a non-diseased control, wherein over-expression of the identified polynucleotide(s) as compared to the non-diseased control is indicative of a diagnosis of hepatocellular carcinoma.

Horne (WO 02/29103) fails to teach this specific method of diagnosis.

The polynucleotide sequence SEQ #2645 in Horne (WO 02/29103), shares 95.6% homology to SEQ ID No. 11. The Horne sequence is disclosed to be down-regulated in metastatic malignant liver (secondary liver cancer). This means that the polynucleotide sequence SEQ #2645 in Horne exhibits the opposite expression pattern compared to elected SEQ ID NO: 11 of the present invention, which is highly up-regulated in HCC (primary liver cancer) (see page 23, line 29 to page 24, line 14 of the instant disclosure). Accordingly, it is clear that the sequence in Horne is related to a different form of cancer than that of the claimed invention.

Therefore, in contrast to the specific method for diagnosing hepatocellular carcinoma of the present invention using SEQ ID NO: 11, sequence SEQ #2645 in Horne is not an optimal hepatocellular carcinoma biomarker. There is no suggestion in Horne to use this sequence for such. In fact, the skilled artisan would not use the sequence in Horne in the claimed method, because the Horne sequence would not be involved in up-regulation, i.e., over-expression, in

hepatocellular carcinoma, as required by the claimed method. Accordingly, the skilled artisan would not select SEQ #2645 (listed among other more than 800 ESTs and genes showing various pattern in primary or secondary liver cancers) to be used in the claimed method, because it not an optimal HCC biomarker involved in up-regulation or over-expression. For this reason, Horne fails to disclose each and every element of the claimed invention. Therefore, Horne fails to anticipate the present invention.

In addition, Applicants again respectfully note that the claims call for methods which require using a "polynucleotide consisting of the polynucleotide sequence of SEQ ID No. 11 or a polynucleotide comprising the polynucleotide sequence of SEQ ID No. 11" and diagnosis by detecting up-regulation.

It is again submitted that Horne fails to disclose or suggest this sequence. Instead, Horne discloses a polynucleotide sequence SEQ #2645 (which shares 95.6% homology to Exon 2 region of clones 5 = SEQ ID No. 11). Such a sequence is not the same sequence as SEQ ID NO: 11. A sequence sharing 95% homology is not the same sequence. Therefore, Horne fails to teach each and every element of the claimed invention. For this reason, Horne fails to anticipate the claimed invention.

In view of the above, the rejection of claims 24, 25 and 34-45 under 35 U.S.C. § 102(b) as anticipated by Horne (WO 02/29103) is untenable and should be withdrawn.

## CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested. If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

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## ATTACHMENT

1. Panzitt et al., Gastroenterology, Vol. 132, No. 1, pp. 330-342 (2007).

GASTROENTEROLOGY 2007;132:330-342

# Characterization of HULC, a Novel Gene With Striking Up-Regulation in Hepatocellular Carcinoma, as Noncoding RNA

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***Background & Aims:*** **Recent studies have highlighted the role of noncoding RNAs (ncRNAs) in carcinogenesis, and suggested that this class of genes might be used as biomarkers in cancer. We searched the human genome for novel genes including ncRNAs related to hepatocellular carcinoma (HCC).** ***Methods:*** **An HCC-specific gene library was generated and screened for deregulated genes with 46 HCCs, 4 focal nodular hyperplasias, and 7 cirrhoses utilizing cDNA arrays. Sequencing of library clones identified a novel ncRNA as the most up-regulated gene in HCC. This gene was also cloned from different monkeys and characterized by quantitative RT-PCR, Northern blot analysis and in situ hybridization. Structural and functional studies included comparative sequence and protein expression analyses, quantitative RT-PCR of polysomal preparations, and siRNA-mediated knockdown experiments.** ***Results:*** **The most up-regulated gene in HCC named *h*ighly *up-regulated* in *l*iver *c*ancer (HULC) was characterized as a novel mRNA-like ncRNA. HULC RNA is spliced and polyadenlyated, and resembles the mammalian LTR transposon 1A. It does not contain substantial open reading frames, and no native translation product was detected. HULC is present in the cytoplasm, where it copurifies with ribosomes. siRNA-mediated knockdown of HULC RNA in 2 HCC cell lines altered the expression of several genes, 5 of which were known to be affected in HCC, suggesting a role for HULC in post-transcriptional modulation of gene expression.** ***Conclusions:*** **HULC is the first ncRNA with highly specific up-regulation in HCC. Because HULC was detected in blood of HCC patients, a potential use as novel biomarker can be envisaged.**



Noncoding RNAs (ncRNAs) have emerged as a new class of functional transcripts in eukaryotic cells, and were grouped into 3 subclasses according to their number of nucleotides.[1,2] The growing class of micro-RNAs (mRNAs) (21–25 nt) has been related to cell differentiation and cancer in recent publications.[3–5] Small ncRNAs with a length of 100–200 nt are commonly found as translational repressors, and long ncRNAs (>10.000 nt) are involved in gene silencing.[2] According to their structural features, these 3 subclasses of heterogeneous transcriptional units can be further subcategorized. mRNA-like ncRNAs, for example, usually lack extensive open reading frames (ORF) and are, therefore, difficult to predict from genomic sequences. In general, they are more than 200 nt long and, in most of the cases, they are spliced and polyadenylated.[6]

Recent studies imply ncRNAs in the regulation of gene expression by a variety of mechanisms such as RNA interference, gene silencing, imprinting, and DNA demethylation, indicating that this novel class of transcripts plays a central role in development and cell differentiation,[6] although to most ncRNAs no specific function has been ascribed. Increasing evidence relates changes in expression levels of ncRNAs to complex diseases such as cancer.[7–10] For PCGEM,[11] and DD3,[12] for example, a tumor-associated overexpression in prostate cancer was found, implicating these ncRNAs in prostate tumorigenesis.[13] BC200 RNA overexpression has recently been correlated with the progression of breast tumors and proposed as a new molecular marker for breast carcinomas.[14] Increased expression of the MALAT-1 gene indicates a worse clinical outcome in lung cancer patients, and thus further emphasizes the potential role of ncRNAs in tumorigenesis.[8]

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide, and affects approximately half a million people yearly.[15] As a multifactorial genetic and epigenetic disease with a complex etiology, most HCCs originate on the basis of long-term liver injury typically caused by chronic hepatitis C or B virus infections, alcoholic liver disease, aflatoxin exposure, or a variety of inherited metabolic diseases.[16] The

*Abbreviations used in this paper:* FNH, focal nodular hyperplasia; HCC, hepatocellular carcinoma; HULC, highly up-regulated in liver cancer; mRNA, micro-RNA; ncRNA, noncoding RNA; ORF, open reading frame; RACE, rapid amplification of cDNA ends.


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majority of these chronic liver diseases lead to the development of liver cirrhosis, which promotes HCC formation. Besides the highly malignant HCC, several other tumor types also occur in the liver. The most common benign tumor-like lesion in the liver is focal nodular hyperplasia (FNH), which is characterized by polyclonal proliferation of hepatocytes and immature ductular cells that are considered to be related to liver progenitor cells.[17]

Based on the increasing evidence for a relationship between ncRNAs and tumor formation and progression, we performed a genome-wide search for novel transcripts, which are associated with the molecular pathogenesis of HCC, but not properly represented in gene collections and commercially available platforms. Utilizing HCC-specific gene libraries and cDNA microarrays, we identified a novel ncRNA as the most up-regulated gene in the HCCs investigated and named it HULC (*highly up-regulated* in *liver* cancer). Due to its striking expression pattern, we performed a detailed characterization of this first ncRNA specifically associated with HCC.

## Materials and Methods

### *Human Tissues*

Human tissue samples were retrieved from the biobank at the Institute of Pathology, Medical University of Graz, Austria. Tissues obtained from surgically resected tumors and adjacent non-neoplastic tissue or from explanted livers were either snap frozen in methyl butane precooled with liquid nitrogen within 20 minutes after operation or fixed in phosphate-buffered (pH 7.4) 4% formaldehyde solution and embedded in paraffin. The study was approved by the ethics committee of the Medical University of Graz, Austria.

### *HCC–cDNA SSH Library Construction*

Total RNA was isolated from 3 HCC samples and from 3 non-neoplastic liver samples using Trizol reagent (Invitrogen, Carlsbad, CA) and pooled. PolyA+-RNA was prepared using Oligotex mRNA spin columns (Qiagen, West Sussex, UK). cDNA synthesis and subtraction were performed using the PCR-Select cDNA Subtraction Kit (BD-Biosciences-Clontech, Mountain View, CA). Subtracted cDNAs were cloned into pCRII (Invitrogen) and transformed into *Escherichia coli* XL-1Blue (Invitrogen).

### *cDNA Microarray Design*

For microarray generation, 2304 colonies containing 960 up- and 960 down-regulated cDNAs plus 384 normalized colonies (where the subtractive step was omitted) were selected from the HCC cDNA library. Additionally, 3514 IMAGE cDNA clones representing genes with well-characterized roles in signaling, cell cycle regulation, apoptosis, tissue remodeling, angiogenesis, and immune reactions were purchased from the RZPD (German Resource Center for Genome Research, Heidelberg, Germany). Four plant clones, cab, tim, xcp2, and rbcl (Stratagene, La Jolla, CA) were included as internal standards for quality control. All 5832 individual clones were PCR-amplified and processed according to standard protocols[18] and printed onto Corning CMT-GAPS™ aminosilane coated glass microscope slides (Corning Life Sciences, New York, NY) using a GMS 417 Arrayer (Genetic MicroSystems, Woburn, MA).

### *cDNA Microarray Hybridization and Analysis of Human Livers*

Frozen tissue sections were dissected under the microscope. Fifty to 100 mg of tissue were used for RNA isolation with Trizol reagent (Invitrogen). Microarray hybridization experiments included a non-neoplastic liver pool (n = 3) as reference. In a direct labeling reaction, 20 μg of total RNA of each sample were spiked with different amounts of *Arabidopsis* mRNAs (Stratagene). The RNAs were fluorescently labeled with either Cy 3 (reference) or Cy 5 dCTP (test sample) by reverse transcription. Probes were hybridized to the chip at 42°C for 16 to 20 hours[18] and microarrays were scanned using a GMS 418 Array Scanner (Genetic MicroSystems). Fluorescent images were analyzed by ImaGene 4.1 and 4.2 software (BioDiscovery, El Segundo, CA). Photomultiplier tube and laser value for scanning were calibrated over all spots in both the Cy 3 and Cy 5 channels. After local background correction by using GeneSight 3.0 software (BioDiscovery), ratios were calibrated by applying normalization factors calculated from the mean intensities over all spots. According to their spike-in ratios, the normalization drift in the 2 fluorescence channels was evaluated. Genes were hierarchically clustered using Genesis software (http://genome.tugraz.at).

### *Statistical Analysis*

Statistical significance of defined subgroups was tested by the Mann–Whitney *U*-test. The same test was used to compute *P* values for testing groups with PCR. In Figures 1C and *D*, 6, and 7 the error bars represent the standard errors of mean (SEM).

### *Rapid Amplification of cDNA Ends (RACE) Library Construction*

The Smart RACE (rapid amplification of cDNA ends) cDNA Amplification kit (BD-Biosciences-Clontech) was used for the construction of 5′ and 3′ RACE libraries from polyA RNA, from total RNA extracted from a pool of HCC tissue and from total RNA primed with multiple gene-specific primers.

### *Data Base Accession Number*

The nucleotide sequence for the HULC region has been deposited into the Genbank data base with the accession number AY914050.

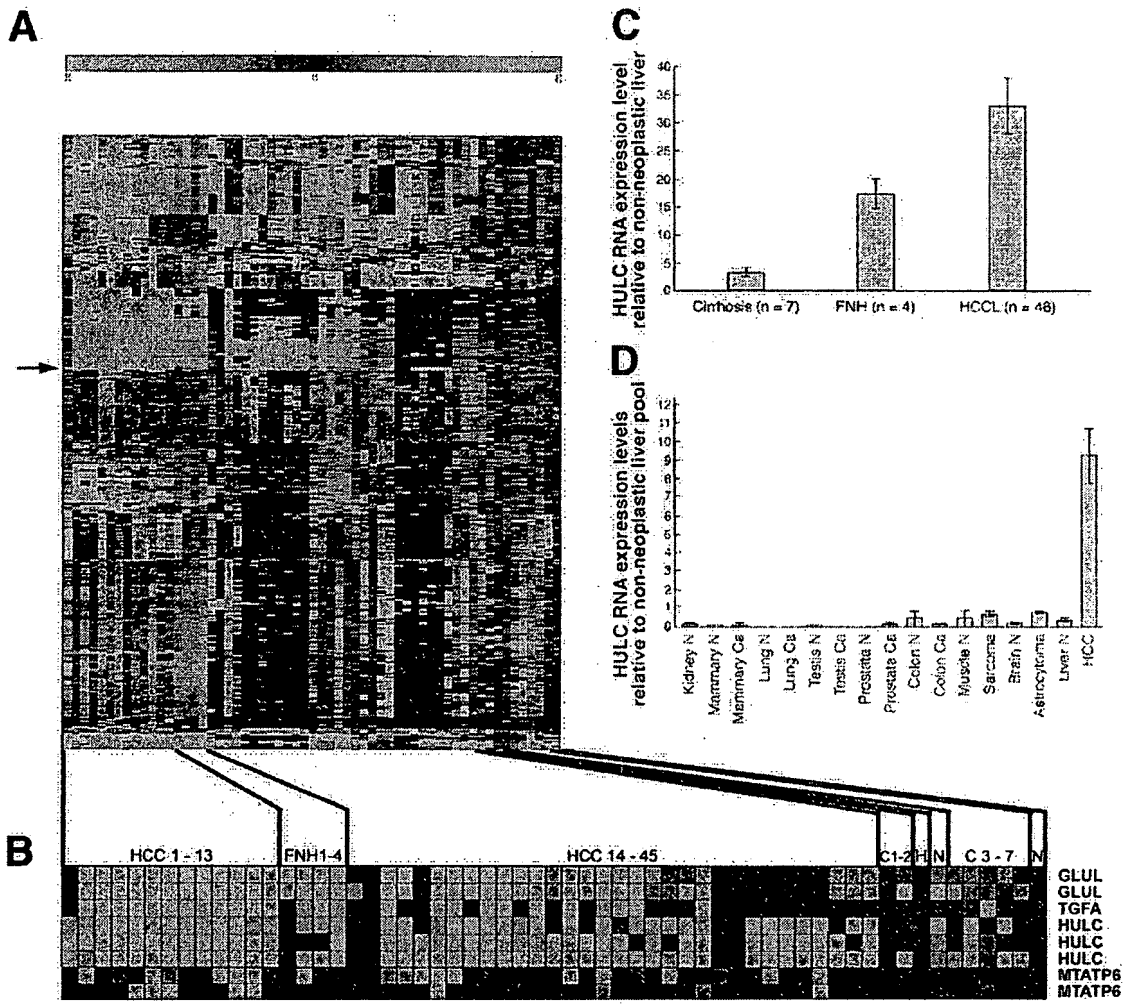


**Figure 1.** Microarray gene cluster analysis. (*A*) mRNA expression profile of 6912 clones (*rows*) from 46 HCCs, 4 focal nodular hyperplasia (FNH) tissue samples, 7 cirrhoses, and 2 non-neoplastic liver samples (*columns*). The genes were hierarchically clustered using Genesis software (http://genome.tugraz.at). The arrow indicates the HULC cluster. (*B*) Magnification of the HULC cluster. Three independent HULC clones are shown in the same cluster as glutamate-ammonia ligase, tumor necrosis factor alpha, and mitochondrial ATPase 6. *H*, hepatocellular carcinoma (HCC); *C*, cirrhosis; *F*, focal nodular hyperplasia (FNH); *N*, non-neoplastic liver, different samples are indicated by consecutive numbers. (*C*) Statistical analysis of microarray data reveals the significant overexpression of HULC in HCC (32.7 ± 5.0; $P = .016$) and FNH (17.1 ± 2.7; $P = .18$) compared with the non-neoplastic liver pool. Error bars represent the standard errors of mean (±SEM). (*D*) Quantitative RT-PCR analysis of HULC RNA expression levels in various non-neoplastic tissues and corresponding tumors. The expression levels of the individual samples are related to the non-neoplastic liver pool (=1). At least 3 independent specimens per tissue sample were analyzed. Fold change values lower than 0.03 (corresponding to Taqman cycle 36) were considered not detectable (n.d.). The up-regulation of HULC is highly significant (9.2 ± 1.5-fold, $P < .005$) and confirms the microarray results. Error bars represent the standard errors of mean (±SEM).



### *Northern Blot Analysis*

Ten micrograms of total RNA were separated on a 1.2% MOPS gel and transferred to a Hybond-N+ membrane (GE Healthcare-Amersham Biosciences, Little Chalfont, UK) by capillary blot. The HULC probe corresponding to nucleotides 99-425 (GenBank accession #AY914050) was radioactively labeled with [$\alpha$-$^{32}$P]dCTP (Amersham Biosciences) using the Rediprime II random prime labeling kit (Amersham Biosciences). ULTRAhyb buffer (Ambion, Cambridgeshire, UK) was used for both prehybridization and hybridization, and signals were detected by autoradiography.

### *Preparation of RNA from Patient Blood and Corresponding Tissue Samples*

For the preparation of peripheral blood mononuclear cells from blood samples, for which informed consent was given, 5 mL of peripheral venous blood was collected in 5 mL vacutainer ethylenediamine tetraacetic acid (EDTA) tubes (BD Pharma, Le Pont-De-Claix, France). Samples were diluted 1:1 in balanced salt solution and subjected to centrifugation in a Ficoll Paque Plus (Amersham Biosciences) gradient in a Sorvall RC-5B centrifuge for 40 minutes at 1400 rpm and 18°C. RNA from cell pellets prepared from the interphase by addi-

tion of 6 mL balanced salt solution and centrifugation for 10 minutes at 700 rpm and 18°C was isolated using the RNeasy Kit (Qiagen). For the extraction of total RNA from frozen tissue samples, 4-μm cryosections were dissected under the microscope. Fifty to 100 mg of tissue were used for RNA isolation with Trizol reagent (Invitrogen) according to the manufacturer's recommendations. Total RNA from paraffin-embedded samples was prepared as described elsewhere.[19]

### *Quantiative RT-PCR*

Using SuperScript II RNase H-reverse transcriptase (Invitrogen), cDNA was synthesized from 1 μg of total RNA prepared from at least 3 independent samples per tissue, including the liver samples from the cDNA array. Applied Biosystems' Universal Taqman mastermix or SYBRgreen mastermix were used with 40 ng of template cDNA per reaction, and samples were analyzed in triplicates. Standard curves were generated in duplicate with 50, 10, 2, and 0.4 ng cDNA, and the values were normalized against GAPDH and/or β-actin. The following primers were used: HULC forward primer: 5′-atctgcaagccaggaagagtc-3′; HULC probe: 5′-FAM-ccagaccatgcaggaactctgatcgtggac-TAMRA-3′; HULC reverse primer: 5′-cttgcttgatgctttggtctgt-3′. GAPDH forward primer: 5′-ccacatcgctcagacaccat-3′; GAPDH probe: 5′-FAM-caaatccgttgactccgaccttca-TAMRA-3′; GAPDH reverse primer: 5′-accaggcgcccaatacg-3′; β-actin forward primer: 5′-aaggccaaccgcgagaagat-3′; β-actin probe: 5′-FAM-ccatgtacgttgctatccaggctgtgctatcc-TAMRA-3′; β-actin reverse primer: 5′-gtcaccggaatccatcacga-3′ (all primers were obtained from MWG biotech, Ebersberg, Germany). After normalization to the housekeeping gene, RNA quantities were shown as fold overexpression.

### *Radioactive In Situ Hybridization*

Radioactive in situ hybridization was performed as previously described.[20] The radioactive probe was generated by in vitro transcription (antisense transcript: nucleotide 95–455 of HULC, GenBank accession #AY914050; sense transcript: nucleotide 92–426, GenBank accession #AY914050). Slides were covered in photographical emulsion K2 (Ilford Ltd., Cheshire, United Kingdom) and stored at 4°C. After 10 days of exposure, the slides were developed with a Kodak D19 developer and counterstained with hematoxylin.

### *Preparation of Ribosome Bound/Free RNA*

Hep3B cells (ATTC #HB-8064) were lysed in NP-40 buffer (0.5% NP-40, 10 mmol/L Tris-HCl pH 8.0, 140 mmol/L NaCl, 1.5 mmol/L $MgCl_2$), supplemented with 480 U/mL RNasin, 150 μg/mL cycloheximide, 20 mmol/L DTT, and 1 mmol/L PMSF. After pelleting the nuclei, mitochondria and membrane particle samples were spun on a 15%–40% sucrose gradient in an ultracentrifuge (Sorvall OTD65B) for 2 hours at 38,000 rpm. Fractions (620 μL) were taken from the gradient and phenol–chloroform extracted. Following LiCl precipitation, RNA preparations were monitored on agarose gels. cDNA was synthesized from 1 μg RNA and subjected to quantitative RT-PCR analysis.

### *Immunoblot*

Cells were lysed in RIPA buffer (50 mmol/L Tris pH 7.6, 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing 2 μg/mL leupeptide/pepstatin/aprotinine (Roche, Basel, Switzerland), 1 mmol/L DDT and 1 mmol/L PMSF. Protein lysate, 15 μg per lane, was separated on 10%–20% Tris-glycine gels and transferred onto a Hybond-P+ membrane (Amersham Biosciences) with a semidry blotter (Amersham Biosciences). Membranes were incubated sequentially with the primary and secondary antibodies for 1 hour each. The following antibodies were used: anti-His (Dianova, Hamburg, Germany), anti-FLAG (Sigma Diagnostics, St. Louis, MO), anti-GFP (Zymed, South San Francisco, CA), swine antirabbit HRP conjugated (Dako, Glostrup, Denmark), rabbit antimouse HRP conjugated (Dako), rabbit anti-HULC peptide antibodies (a: 0.15 mg/mL; b: 0.1 mg/mL) to the putative peptide sequences: (a) PREDTARPQSASQE, (b) DTARPQSASQEESSR. Signals were detected with the ECL kit (GE Healthcare-Amersham Biosciences).

### *Plasmid Transfections*

Hep3B cells were cultured in DMEM medium (Invitrogen, Gibco cat #41965-039) supplemented with 1% Penicillin/Streptomycin (Invitrogen, Gibco, cat #15140-122), 1% L-glutamine (Invitrogen, Gibco cat #25030-024), and 10% fetal calf serum (Gibco, cat #10270-106) at 37°C in 95% $H_2O$ and 5% $CO_2$. Plasmids were transfected with Lipofectamine reagent or Lipofectamine 2000 reagent (Invitrogen).

### *siRNA-Mediated Knockdown of HULC*

$4 \times 10^5$ cells/well HepG2 (ATTC #HB-8065) or $2 \times 10^5$ cells Hep3B (ATTC #HB-8064), respectively, were seeded into 6-well plates the day before transfection in DMEM medium containing 10% fetal calf serum. Chemically synthesized siRNA oligonucleotides were purchased from Qiagen-Xeragon (Germantown, MD) and designed using an siRNA design algorithm applying stringent homology analysis (https://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/CustomSiRnaOrder.aspx). Two siRNA oligonucleotides targeting 2 different sections of the HULC RNA were used for the experiments (target sequence: 5′-aatctgcaagccaggaagagt-3′ for HULC siRNA 1 and 5′-aacctccagaactgtgatcca-3′ for HULC siRNA 2). Controls involved a scrambled (target sequence: 5′-aaccactgccttgatccgaaa-3′) and a nonsilencing control siRNA (#1022076, target sequence: 5′-aattctccgaacgtgtcacgt-3′) as negative controls and

Lamin A+C siRNA (#1022050; target sequence 5′-aactggacttccagaagaaca-3′) as positive control. Transfection was carried out using Oligofectamine (Invitrogen) according to the manufacturer's recommendations. Briefly, cells were washed prior to transfection with DMEM without additives and cultured in 800 μL DMEM per well without additives. For transfection, 10 μL of a 20 mmol/L siRNA oligonucleotide stock (or 10 μL siRNA resuspension buffer for mock transfection controls) diluted in 175 μL Opti-MEM Reduced Serum Medium (Invitrogen) per well were complexed by addition of 2.7 μL Oligofectamine and 12 μL Opti-MEM Reduced Serum Medium. After 6 hours, fetal calf serum concentration was adjusted to 10%. Cells were harvested 24 hours after transfection. Cells of a 6-well plate were pooled and subjected to RNA isolation using Trizol reagent (Invitrogen). One microgram of total RNA was reversely transcribed and the degree of knock down was determined by quantitative RT-PCR. After RNeasy clean-up (Qiagen), samples were subjected to microarray hybridization.

### *Microarray mRNA Expression Analysis*

The quality of RNA prepared from siRNA-treated cells was evaluated using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Samples with a RNA Integrity Number value above 9 were subjected to labeling (35 μg of total RNA per sample) following the Applied Biosystems Chemiluminescent Labeling protocol (Applied Biosystems, Foster City, CA). The directly labeled cDNA product was hybridized to Human Genome Survey Microarrays v02 (Applied Biosystems) covering approximately 28,000 genes according to the manufacturer's instructions. Microarrays were analyzed using the AB1700 Chemiluminescent Microarray Analyzer. For each cell line and for each knockdown experiment 2 biological replicates were analyzed.

### *Microarray Data Processing and Statistics*

Array images were processed using the Applied Biosystems 1700 Array Scanner software. Spot normalization and statistical analysis were used to determine significantly ($P < .05$) up- or down-regulated genes affected by siRNA-mediated knockdown. Expression values were normalized across arrays by quantile normalization using R Script and the Bioconductor software (http://www.bioconductor.org/). Significantly up-regulated genes ($P < .05$) were subjected to functional classification using PANTHER software (Applied Biosystems) to evaluate pathways and biological processes affected by the siRNA knock-down.

## Results

### *HULC Is Specifically Expressed in Hepatocytes and Highly Up-regulated in HCC*

For a genome-wide gene expression profiling approach, we generated HCC-specific cDNA libraries by subtractive suppressive hybridization.[21] Clones from the HCC cDNA library in conjunction with well-known cancer-related and control genes were used for the production of an HCC-specific cDNA microarray containing a total of 6912 cDNA clones. Tissue samples of 46 HCCs, 4 FNHs, 7 cirrhoses, and 2 non-neoplastic livers were compared with a pool of non-neoplastic liver samples (Figure 1*A*; full dataset will be provided as supplemental material on our website). The most prominent cluster of up-regulated genes contained a previously unidentified EST together with transforming growth factor alpha and glutamate-ammonia ligase, 2 genes that are known to be highly up-regulated during the pathogenesis of human HCC (Figure 1*B*).[16,22,23] Up-regulated 33-fold on average ($32.7 \pm 5.0$, $P = .016$) over the non-neoplastic liver pool in 76% of HCCs, and represented by 3 independent HCC-specific library clones, this EST was named HULC. HULC expression levels were also, but to a lesser extent, up-regulated ($17.1 \pm 2.7$-fold) in FNH ($P = .188$). In contrast to the situation in liver tumors, HULC was only slightly up-regulated in cirrhotic liver tissue ($3.3 \pm 0.7$-fold, $P < .001$) (Figure 1*C*).

To determine whether HULC is commonly overexpressed in neoplastic tissues of various organs, a broad range of normal tissues and their corresponding carcinomas/sarcomas were tested for HULC RNA expression by quantitative RT-PCR (Figure 1*D*). HULC was barely detectable in most of the normal tissues and not significantly elevated in the majority of the corresponding neoplastic tissues. The slight increase of HULC RNA levels in prostate carcinoma, astrocytoma, and sarcoma, however, was never as striking as in HCC, where the mean HULC RNA expression level exceeded all other normal and neoplastic tissues tested at least by a factor of 12. The quantitative RT-PCR results mirrored the cDNA microarray data, although the levels of up-regulation were generally lower ($9.2 \pm 1.5$-fold, $P < .005$ versus $32.7 \pm 5.0$, $P = .016$-fold in the cDNA microarray). Nevertheless, these results demonstrate a highly specific up-regulation of HULC RNA expression levels in HCC. In situ hybridization revealed the specific and strong expression of HULC RNA in the cytoplasm of HCC, whereas it was not detected in tumor stroma and non-neoplastic liver cells (Figure 2).

### *Characterization of the HULC Transcriptional Unit*

Northern blot analysis identified an HULC transcript with a length of approximately 500 nucleotides. Again, HULC RNA levels were markedly higher in HCC than in non-neoplastic liver (Figure 3*A*), which is consistent with our cDNA microarray and quantitative RT-PCR data. To clone a full-length gene and to localize the HULC transcriptional unit, polyA primed and gene-specifically primed 5′ and 3′ RACE (rapid amplification of cDNA ends) cDNA pools were generated. Sequencing of

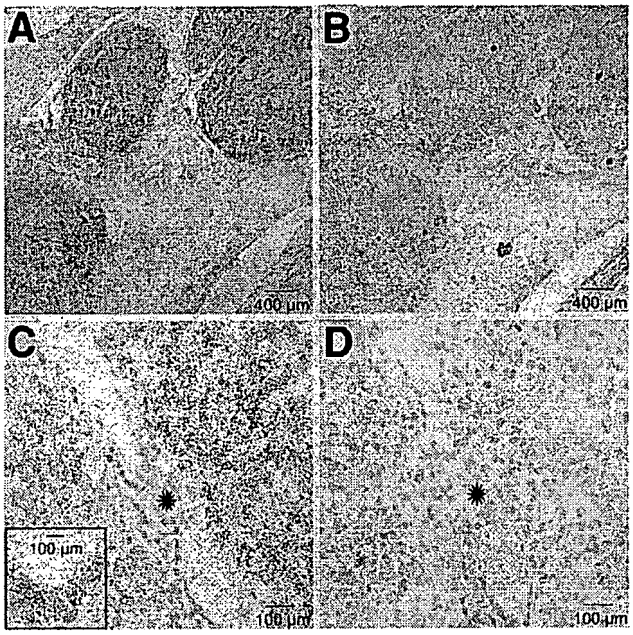


**Figure 2.** Detection of HULC expression in HCC by radioactive in situ hybridization. (*A*) Hybridization with antisense probe shows positive signal in HCC nodules, whereas the surrounding non-neoplastic tissue is negative. (*B*) Hybridization with the sense probe of the corresponding area shown in (*A*). (*C*) Higher magnification of antisense probe hybridization allows localization of the specific signal to the cytoplasm of hepatocellular carcinoma cells. *Insert* shows higher magnification of the same section. (*D*) Corresponding area as shown in (*C*) hybridized with sense probe. Asterisks indicate corresponding tumor stroma areas in (*C*) and (*D*). Scale bar in *A* and *B* equals 400 μm; in *C*, *D*, and *insert* equals 100 μm.

HULC-specific RACE PCR products localized the HULC sequence to a 482-bp region on human chromosome 6p24.3. Computational analysis of the obtained nucleotide sequence data allowed us to determine the structure of the HULC transcriptional unit. The HULC gene includes a single intron and carries 1 canonical and 2 noncanonical polyadenylation signals 21 nt, 32 nt, and 47 nt, respectively, upstream from the polyA tail (Figure 3*B* and *C*). The genomic region harboring the HULC locus is poorly characterized. However, exon 1 mainly consists of an LTR (MLT1A; mammalian LTR transposon 1A), indicating the integration of a retrotransposon at this chromosomal location.[24]

Computer-assisted database comparison revealed that there was, prior to our findings, no corresponding full-length cDNA clone in the publicly available databases. However, as expected from the abundance of the HULC transcript, we identified 5 ESTs corresponding to HULC (UniGene cluster Hs. 214343) in blast searches against the NCBI genomic and EST databases and the UCSC genome browser. Most of these ESTs were isolated either from a pool of fetal liver and spleen cDNA or a mixed pool of cDNAs from 40 tissues including liver.

### *HULC Is Evolutionary Conserved in Primates*

Further in silico searches of public domain databases (NCBI, UCSC, Ensembl) did not identify any HULC homologue in other species, except for a partial homology in chimpanzee for exon 2. To find possible HULC homologues in primates, we cloned and sequenced HULC from 3 Old World monkeys (*Papio ursinus*, *Macaca mulatta*, and *Macaca fasciculata*). Both HULC exons were amplified with low stringency quantitative RT-PCR conditions from liver RNA isolated from these primates using various human primers. Sequencing of the resulting PCR products demonstrated that both HULC exons were highly conserved in these species (1 mismatch/482 bp), whereas the sequences upstream of exon 1 and the introns revealed 18 and 17 mismatches, respectively, in the approximate 300 bp sequenced (Figure 4). In addition, an analysis of CpG dinucleotides was performed.[25] The human HULC exons show a high content of CpG dinucleotides with an incidence of 5 CpG dinucleotides in 482 bp. In contrast, the next CpG dinucleotide 5′ to

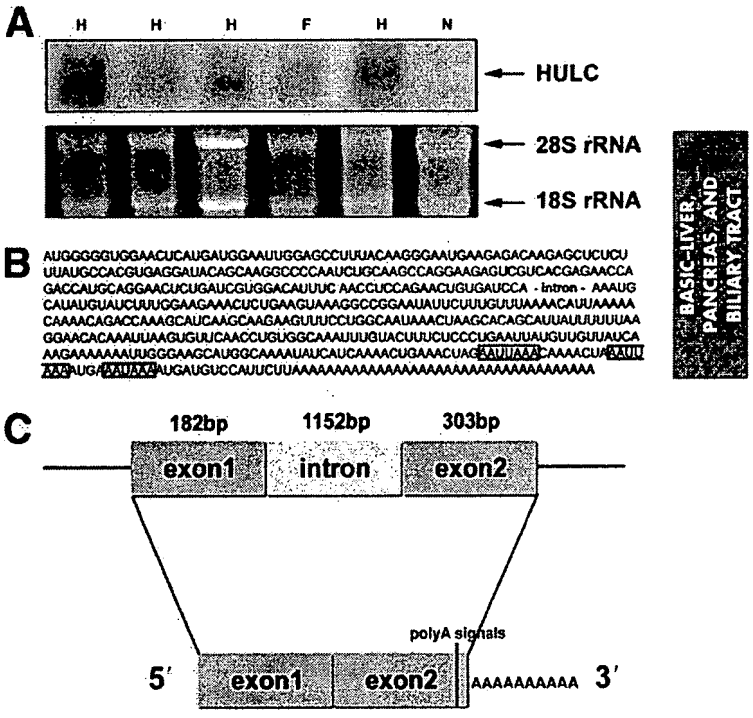


**Figure 3.** Characterization of the HULC transcriptional unit. (*A*) Northern blot analysis of HULC RNA. Ten micrograms of total RNA were loaded per lane. A positive signal at 500 nt indicates specific overexpression in different tumorous tissues. *H*, HCC; *F*, focal nodular hyperplasia (FNH); *N*, non-neoplastic liver. 18S and 28S rRNA were used as a control for the quantity and quality of RNA loaded. (*B*) The HULC gene locus. HULC RNA sequence including the polyA tail showing the location of the intron and the polyadenylation signals (*boxes*). (*C*) Diagram of the HULC gene locus. The HULC gene consists of 2 exons, 182 nt and 303 nt long, with 1 standard AATAAA and 2 noncanonical AATTAA polyadenylation signals occurring 21 nt, 32 nt, and 47 nt, respectively, upstream from the polyA tail.

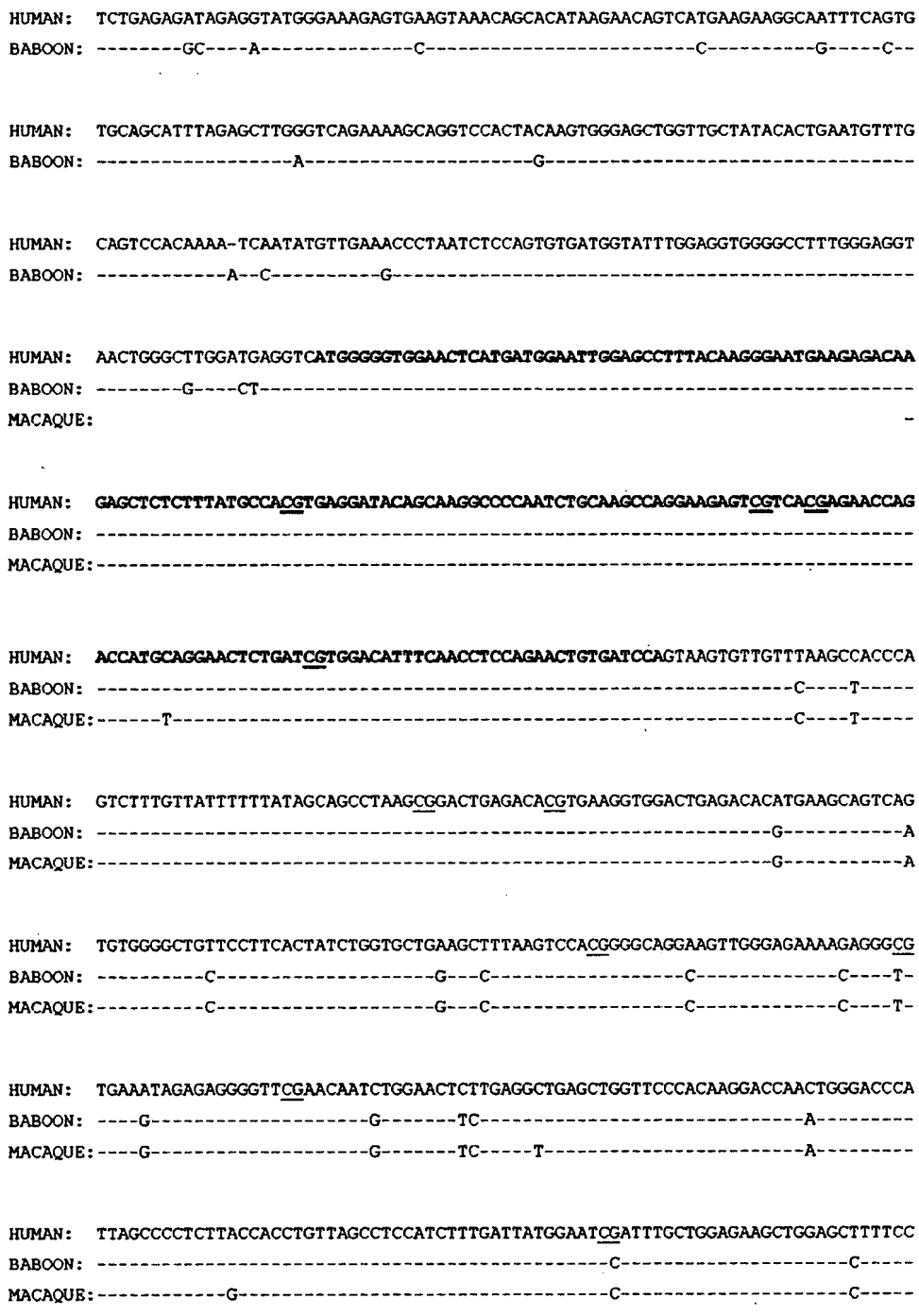


**Figure 4.** HULC is evolutionary conserved in primates. Only the first exon (bold) surrounded by upstream genomic and intronic sequence is shown. Matches between the indicated primate sequences and that of *H sapiens* are indicated by hyphens. Nucleotide replacements are shown at the appropriate location. CpG dinucleotides are underlined.

exon 1 was 808 bp and 3′ to exon 2 was 566 bp from the HULC coding sequence. This indicates high evolutionary conservation of HULC and that it is an active transcriptional unit. Surprisingly, neither mouse nor rat appears to have a HULC homologue. The syntenic chromosomal sequence both upstream and downstream of the presumed HULC transcriptional unit in rodents are highly homologous for several hundred kilobases (84.4% to mouse chromosome 13, 87.0% to rat chromosome 17 for the upstream region; 81.3% and 87.1% for the downstream regions, respectively). However, no homology is apparent for the HULC transcriptional unit itself.

### *HULC May Function as an ncRNA*

One striking feature of the HULC RNA sequence is the high density of stop codons in the small potential reading frames scattered throughout the HULC gene. Furthermore, in silico translation of the HULC RNA did

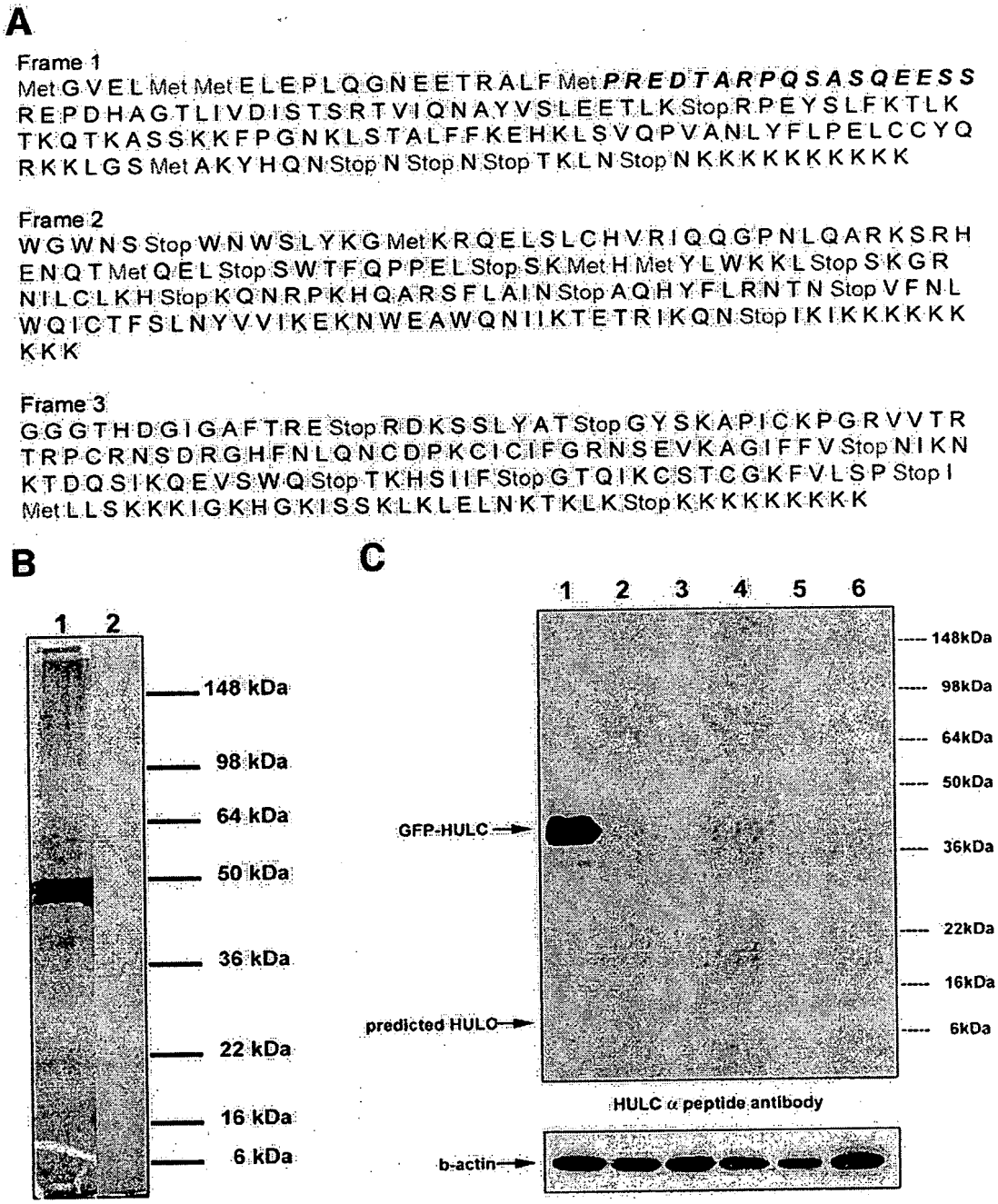


**Figure 5.** Expression of HULC as a fusion protein and in vitro translation. (*A*) The longest potential ORF found in the HULC gene is 72 amino acids (219 nt) long (*frame 1*). The *bold* letters indicate the location of 2 overlapping HULC epitopes that were used for the generation of antipeptide antibodies. (*B*) Western blot of extracts from different HCC tissues expressing high levels of HULC mRNA and from HULC transfected HepG2 cells with an antibody raised against a putative HULC peptide. A specific protein product is detectable only in the C-terminal HULC fusion construct (*lane 1*). Equal amounts of protein were loaded per lane as shown by the β-actin control. (1) GFP-HULC transfected HepG2 cells; (2) GFP transfected HepG2; (3) HCC; (4) HCC; (5) HepG2 cells transfected with nontagged HULC cDNA; (6) mock transfected HepG2 cells.



not indicate the presence of a substantial ORF in the 482 nt-long HULC sequence. The longest predicted ORF with a potentially functional translation initiation site is 219 nt long, and would yield a possible gene product of 72 amino acids (approximately 8 kilodaltons) (Figure 5*A*). However, the deduced amino acid sequence does not contain any known protein motifs nor does it share homologies to other proteins in any species. Even though the lack of an ORF larger than 100 amino acids suggested that the HULC RNA might not be translated,[26] we per-

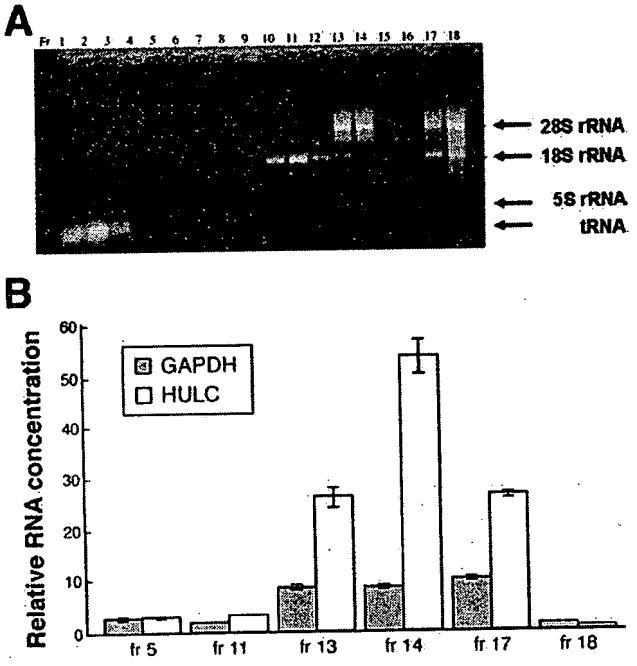


**Figure 6.** Polysomal profile of HULC. (*A*) Polysomal fractions (fr) were isolated after sucrose gradient centrifugation and loaded onto a denaturing (1 × MOPS) RNA gel. The first fractions (1–5) on the gel demonstrate RNA not bound to ribosomes, whereas subsequent fractions represent ribosome bound RNA (fractions 6–18). (*B*) Representative ribosome fractions were examined by quantitative RT-PCR and relative concentrations of HULC and GAPDH RNA were calculated from total RNA standard curves. Both genes showed elevated expression levels in the ribosome-bound fractions starting with fraction 13, where the first complete ribosomes were observed.



formed in vitro translation experiments to determine whether any protein product was generated from the HULC RNA sequence. In vitro translation as well as overexpression of HULC in *E coli* (data not shown) did not yield any protein product detectable on gels designed to resolve proteins smaller than 4 kilodaltons.

Furthermore, we generated polyclonal antisera to 2 peptides deduced from this potential ORF sequence to detect a putative small protein encoded by this ORF. With these antisera, immunofluorescence studies were performed on frozen sections of HCC tissue and on HepG2 and Hep3B hepatoma cell cultures transfected with the putative HULC ORF expression construct. In neither case was a positive signal identified. Moreover, HULC protein could not be detected by immunoblot analysis of extracts from HCCs expressing high levels of HULC RNA or from HepG2 cells transfected with a HULC expression construct (Figure 5*B*). However, when the putative HULC ORF sequence was fused to the C-terminus of GFP or to a FLAG tag, the polyclonal antisera detected the peptide sequence in the fusion protein indicating that antibodies were able to bind to the peptides used for immunization.

### *HULC Is Associated With Ribosomes of Carcinoma Cells*

The cytoplasmic localization of HULC was further confirmed by analysis of subcellular compartments (cytoplasm, nucleus, nucleolus) with quantitative RT-PCR. In line with the results obtained by ISH, the highest concentrations of HULC were detected in the cytoplasm (data not shown). Because 2 previously described human cancer-related ncRNAs, H19 and BC200, were reported to be associated with ribosomes and to regulate translation,[25,27,28] we further investigated whether HULC RNA also localized to the ribosomes. Analysis of RNA isolated from the ribosomal fractions by gel electrophoresis (Figure 6*A*, fractions 6–18) and quantitative RT-PCR analysis of RNA extracted from ribosomal preparations (Figure 6*B*) revealed a predominant presence of HULC RNA in the ribosome fraction.

### *siRNA-Mediated Knockdown of HULC in the Hepatoma Cell Lines Hep3B and HepG2*

To obtain a first insight into a possible role of HULC in hepatocellular carcinogenesis, the effect of siRNA-mediated knockdown of HULC expression was investigated in 2 hepatoma cell lines by genome-wide transcriptional profiling. For that purpose, Hep3B and HepG2 cells were treated for 24 hours with 2 different siRNA oligonucleotides (HULC siRNA 1 and HULC siRNA 2) targeting different sections of the HULC RNA. Two different negative control siRNAs (scrambled HULC and a nonsilencing control) were included in this experiment to monitor the specificity and efficiency of HULC knockdown. Furthermore, siRNA to lamin A + C was used as positive control and for optimization of the transfection procedure (data not shown). RNA from 2 biological replicates of these experiments was subjected to quantitative RT-PCR for quantification of HULC knockdown (Figure 7). Knockdown efficiencies of 67%

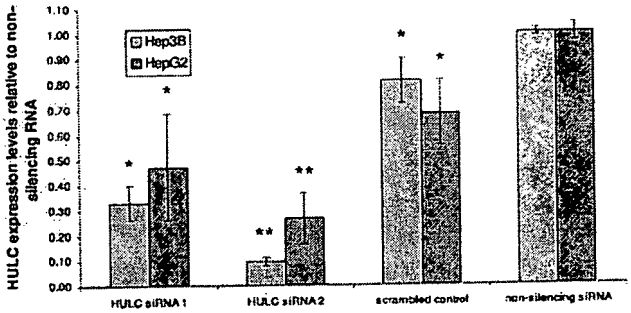


**Figure 7.** Efficiency of siRNA-mediated HULC knockdown in the Hep3B and HepG2 hepatoma cell lines. Cells were treated with 2 different siRNA oligonucleotides and 2 different control siRNAs for 24 hours. HULC RNA expression levels from 2 biological replicates were monitored by quantitative RT-PCR in triplicates, revealing the indicated knockdown efficiencies relative to the nonsilencing control (=1). Error bars present the standard errors of mean (±SEM, n = 6). *$P < .01$, **$P < .05$ versus nonsilencing siRNA control (=1), Student's *t*-test.

**Table 1.** Significant ($P < .05$) Deregulation in Both Cell Lines with Both siRNAs Compared to Both Control siRNAs

| Acc. no. | Gene name | Function |
|---|---|---|
| Upregulation in HULC knockdown | | |
| NM_013275.4 | Ankyrin repeat domain 11 | Unclassified |
| NM_018294.3 | CWF19-like 1, cell cycle control (S. pombe) | Unclassified |
| NM_001260.1 | Cyclin-dependent kinase 8* | Protein phosphorylation, cell cycle control, mitosis |
| NM_001281.2 | Cytoskeleton associated protein 1 | Protein folding, cell structure |
| NM_005265.1 | γ-glutamyltransferase-like activity 4* | Amino acid metabolism, protein modification, proteolysis |
| NM_015710.3 | Glioma tumor suppressor candidate region gene 2 | Tumor suppressor |
| NM_017969.1 | Hypothetical protein FLJ10006 | Unclassified |
| NM_199054.1 | Lymphotoxin beta receptor (TNFR superfamily, member 3)* | Receptor-mediated signal transduction, T-cell immunity, Apoptosis |
| NM_002342.1 | MAP kinase interacting serine/threonine kinase 2* | Protein phosphorylation, hematopoiesis |
| NM_182977.1 | Nicotinamide nucleotide transhydrogenase | Oxidative phosphorylation |
| NM_016122.1 | NY-REN-58 antigen | Unclassified |
| NM_001009555.1 | SH3 domain protein D19 | Unclassified |
| XM_498268 | Similar to 60S ribosomal protein L23a | Protein biosynthesis |
| NM_020123.2 | SM-11044 binding protein | Transport |
| NM_003083.1 | Small nuclear RNA activating complex, polypeptide 2, 45kDa | Unclassified |
| NM_002999.2 | Syndecan 4 (amphiglycan, ryudocan)[a] | Cell adhesion, macrophage-mediated immunity, skeletal development |
| NM_013245.2 | Vacuolar protein sorting 4A (yeast) | Protein metabolism/modification, protein targeting, vesicle transport |
| NM_007152.1 | Zinc finger protein 195 | mRNA transcription regulation, cell proliferation and differentiation |
| NM_014941.1 | Zinc finger, CW type with coiled-coil domain 1 | mRNA transcription regulation, cell proliferation and differentiation |
| Downregulation in HULC knockdown | | |
| NM_017955.2 | Cell division cycle associated 4 | Unclassified |
| NM_018170.2 | Hypothetical protein FLJ10656 | Unclassified |
| NM_004549.3 | NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2 | Oxidative phosphorylation |
| AK025133.1 | Nuclear ubiquitous casein kinase/cyclin-dependent kinase substrate | Mitosis |

Genes are grouped together with their GenBank accession numbers ("Acc. no.") and annotated function according to PANTHER.
[a]Genes that have been described in the context of liver cancer. Significant ($P < .05$) deregulation in both cell lines with both siRNAs compared to both control siRNAs.



and 90% were obtained for HULC siRNA 1 and 2 in Hep3B cells, whereas HULC knockdown in HepG2 cells was less efficient and reduced HULC expression by 53% and 73% compared with the respective nonsilencing controls. Importantly, an activation of the double-stranded RNA-triggered IFN-associated antiviral pathways did not occur under these experimental conditions, because the expression of the sensitive marker genes 2′-5′-oligoadenylate synthetase and interferon-inducible transmembrane protein 1[29] was not affected (data not shown).

For genome-wide transcriptional profiling, RNAs prepared from 2 biological knockdown replicates in Hep3B and HepG2 cells obtained with the 2 different siRNAs against HULC and the 2 different control siRNAs were subjected to microarray analysis. Table 1 shows the list of genes that were significantly up- or down-regulated ($P < .05$) in HepG2 as well as in Hep3B cells compared to the 2 controls. These genes—of which several have already been described in the context of liver cancer—fulfill different biological functions. To explore possible common features, we searched for homologies between these sequences and between HULC and these sequences (including the 5′-UTRs), which did not reveal any striking homologies.

### *Detection of HULC RNA in Peripheral Blood Cells of HCC Patients*

In a pilot experiment, blood samples obtained from healthy volunteers (n = 9), patients with liver cirrhosis (n = 10), and 4 HCC patients (n = 4) were tested by quantitative RT-PCR for the presence of HULC RNA in cells of peripheral blood. The HULC/β-actin ratios obtained for 3 out of 4 individual HCC patients revealed markedly increased concentrations of HULC RNA (Figure 8): HULC expression levels in blood sample 1 were at least 10 times, in blood sample 3 at least 17 times and in blood sample 4 at least 30 times higher than in the pool of patients without any known liver disease. For 2 of the HCC patients (donors of blood sample 1 and blood sample 3), cryo-preserved, and paraffin-embedded tissue samples from surgically resected tumors and corresponding non-neoplastic liver tissue were available for a correlation of HULC expression in blood with HULC expression in liver tissue. HCC areas were dissected from the

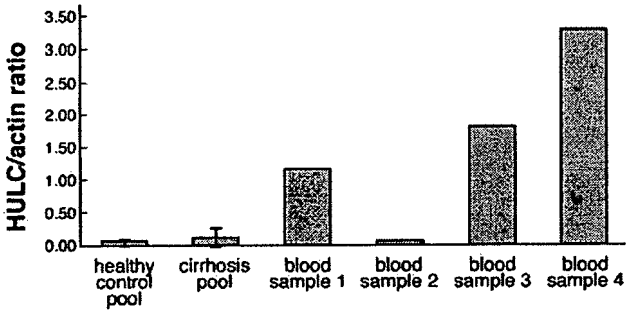


**Figure 8.** Detection of HULC RNA in cells of peripheral blood from HCC patients. Total RNA was extracted from peripheral blood cells from healthy volunteers (=healthy control pool, n = 9), patients suffering from cirrhosis (=cirrhosis pool, n = 10) and HCC patients (=blood samples 1–4) and tested for HULC expression by quantitative RT-PCR. Samples are plotted against the calculated HULC/β-actin ratios. Minimum increases in HULC expression of 10-, 17-, and 30-fold, respectively, were detected in blood samples of 3 out of 4 HCC-patients compared to non-neoplastic controls. Corresponding paraffin-embedded tissue samples were available from 2 of these patients (blood samples 1 and 3) for a correlation of HULC expression in blood and tissue. Error bars present the standard errors of mean (±SEM).

surrounding non-neoplastic and cirrhotic liver tissue under the microscope and HULC expression levels in both samples were compared by quantitative RT-PCR. HULC expression in HCC tissue was 5 and 10 times, respectively, higher than in the surrounding non-neoplastic liver tissue (data not shown).

## Discussion

Using an HCC-specific cDNA microarray platform, we identified HULC, a novel mRNA-like ncRNA, as 1 of the most up-regulated genes in HCC. The levels of HULC RNA expression and up-regulation exceeded well-established, cancer-related genes analyzed in our study as well as those identified in other gene expression profiling studies.[30–32] Examples of such genes are the E2F targets proliferating cell nuclear antigen and histone 1, the proliferation-associated genes cyclin D1, and c-*jun*, the hepatocyte growth factor receptor (data not shown), as well as transforming growth factor alpha and glutamate-ammonia ligase, the latter being a target of the wnt-signaling pathway (Figure 1, See supplemental material online at http://pathologie.meduni-graz.at/Pathologie/laboratorium_fuer_molekularpathologie.htm).[32–36] Up-regulation of a HULC-related EST (UniGene Hs. 214343) in the Asian population was independently described by Okabe et al in an earlier study,[37] indicating that HULC is not restricted to HCC in Caucasians, as investigated in our study. As such a striking expression pattern has not been reported for any other disease-associated ncRNA, we performed a detailed characterization of HULC.

The characterization of HULC as an ncRNA is based on several experimental approaches and findings: (1) The HULC sequence does not clearly identify a substantial open reading frame, (2) in vitro translation did not generate any detectable protein product, (3) polyclonal antibodies raised against a hypothetical HULC protein did not reveal a positive reaction in HCC tissues expressing a high level of HULC RNA, (4) neither prokaryotic nor eukaryotic protein expression systems were able to produce a HULC-encoded protein, and (5) a protein was detectable with the antipeptide antibodies only if the HULC sequence was fused to the C-terminus and not to the N-terminus of GFP. A possible explanation why HULC is only translated as a C-terminal fusion protein was provided by structural analyses of HULC RNA using the mfold program (www.bioinfo.rpi.edu/applications/mfold/old/rna/), which revealed pronounced secondary structures usually not found in translated mRNAs (data not shown).

In this context it was also important to demonstrate the full length of the HULC cDNA. The size of clones obtained by different RACE protocols was compatible to the RNA length detected by Northern blotting. Furthermore, cloning of HULC homologue genes in 3 Old World monkey species showed high conservation of the HULC sequence within the exons, but not in the further 5′-region and intron, which indicates the size and location of functional relevant domains within the HULC RNA (Figure 4). Evidence for the origin of the HULC gene is based on the findings that the first exon of the HULC sequence mainly consists of an LTR (MLT1A; mammalian LTR transposon 1A).[24] We hypothesize that a mammalian LTR transposon retroposed from an active mastergene.[25] Interestingly, a mouse homologue of HULC was not detectable; however, the sequences directly surrounding the HULC gene locus bear homologies to both mouse and rat syntenic chromosomes. This suggests that HULC has been created by retroposition approximately 25 million years ago, before the Old World monkeys radiated from primates.[25] As described by Brosius,[38] retronuons are frequently converted into regulatory elements that may alter expression or processing of target genes.[39] A similar mechanism might be pertinent for HULC. The biological relevance of this region is further supported by the evolutionary conserved presence of CpG dinucleotides. When genetic elements transpose, CpG dinucleotides are most rapidly eliminated due to methylation of the cytosine. A conservation of the CpG sequence is indicative of a functional significance of this element (J. Brosius, personal communication, March 2004). Furthermore, sequences comprising more than 2 kb upstream of the HULC locus were analyzed for their CpG content involving the programs CpG plot, CpG Island Searcher, CpG report, and GrailEXP. However, these programs did not highlight the presence of typical CpG dinucleotides, which are targets for methylation, in the 5′-upstream region, thus underlining the functional relevance of conserved CpG dinucleotides in the HULC sequence itself.

First clues about the function of HULC were obtained from siRNA-mediated knockdown of HULC in Hep3B and HepG2 cells, which resulted in a significant up- and down-regulation of several genes, some of which have already been described in the context of liver cancer. This indicates that there may not only be 1 single HULC target gene, but rather suggests a more general regulatory role for HULC. The association of HULC with the ribosome could provide a hint to its mode of action, as it is reminiscent of the localization of siRNAs, which have recently been found to be physically associated with the ribosome. At the ribosome, siRNAs form an 80S complex that is able to cleave targeted mRNAs and thus can act as translational repressors.[41] In contrast to such small ncRNAs, for which a localization to the ribosome has already been described[2] and which have already gained interest as genetic elements actively involved in carcinogenesis, we describe HULC here as an example for the association of an mRNA-like ncRNA with ribosomes. Preliminary studies revealed no significant homologies between HULC and its putative target sequences, thus indicating that the effect of HULC on its targets may not be based on direct RNA-RNA interactions. Whether the mechanism by which HULC affects mRNA concentrations of targets constitutes a common regulatory principle remains to be resolved.

HULC expression was also increased in FNH, which is a benign liver tumor resulting from increased polyclonal proliferation of hepatocytes due to an uncharacterized proliferation stimulus.[17] Although FNH does not progress to HCC, there are several biological processes affected in FNH, which also play a role in carcinogenesis (ie, angiogenesis, alterations in cell metabolism, increased cell proliferation, activation of a precursor cell compartment, and tissue remodeling). The up-regulation of HULC expression in FNH consequently also correlates HULC expression with benign tumor-like lesions, suggesting that HULC contributes to modulation of gene expression already very early in tumorigenesis rather than being involved in the promotion of the highly malignant HCC phenotype.

Unfortunately, because there is no mouse homologue of HULC, there are currently no in vivo models available to study this mechanism in more detail. However, the association of HULC with the polysomes and our knockdown data suggest a role of HULC in the post-transcriptional control of gene expression.

The recent identification of new classes of ncRNAs implicated in important steps of cancer formation and progression reinforces the role of these transcripts in the process of tumorigenesis and suggests several potential targets for drug discovery and biomarkers for early diagnosis. DD3, for example, is the most prostate cancer-specific ncRNA described,[12] and forms a very sensitive and specific marker for the detection of tumor cells.[40] BC1 is deregulated in breast cancer[9] and BC200 RNA overexpression was recently evaluated as a new molecular marker for a poor prognosis in breast carcinomas.[14] In this context, the potential role of HULC as a novel biomarker is based on its striking expression pattern and underlined by the fact that HULC RNA can be detected in the blood of HCC patients and in corresponding tissue samples by RT-PCR.[27,33]

## References


1. Mattick JS. Non-coding RNAs: the architects of eukaryotic complexity. EMBO Rep 2001;2:986–991.
2. Costa FF. Non-coding RNAs: new players in eukaryotic biology. Gene 2005;357:83–94.
3. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–838.
4. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet 2005;37:766–770.
5. Jopling C, Yi M, Lancaster A, Lemon S, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. Science 2005;309:1577–1581.
6. Erdmann VA, Szymanski M, Hochberg A, de Groot N, Barciszewski J. Collection of mRNA-like non-coding RNAs. Nucleic Acids Res 1999;27:192–195.
7. Tam W, Ben-Yehuda D, Hayward WS. Bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its non-coding RNA. Mol Cell Biol 1997;3:1490–1502.
8. Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H, Muller-Tidow C. MALAT-1, a novel non-coding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 2003;22:8031–8041.
9. Chen W, Bocker W, Brosius J, Tiedge H. Expression of neural BC200 RNA in human tumours. J Pathol 1997;183:345–351.
10. Li J, Witte DP, Van Dyke T, Askew DS. Expression of the putative proto-oncogene His-1 in normal and neoplastic tissues. Am J Pathol 1997;150:1297–1305.
11. Srikantan V, Zou Z, Petrovics G, Xu L, Augustus M, Davis L, Livezey JR, Connell T, Sesterhenn IA, Yoshino K, Buzard GS, Mostofi FK, McLeod DG, Moul JW, Srivastava S. PCGEM1, a prostate-specific gene, is overexpressed in prostate cancer. Proc Natl Acad Sci U S A 2000;97:12216–12221.
12. Bussemakers MJ, van Bokhoven A, Verhaegh GW, Smit FP, Karthaus HF, Schalken JA, Debruyne FM, Ru N, Isaacs WB. DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. Cancer Res 1999;59:5975–5979.
13. Petrovics G, Zhang W, Makarem M, Street JP, Connelly R, Sun L, Sesterhenn IA, Srikantan V, Moul JW, Srivastava S. Elevated expression of PCGEM1, a prostate-specific gene with cell growth-promoting function, is associated with high-risk prostate cancer patients. Oncogene 2004;23:605–611.
14. Iacoangeli A, Lin Y, Morley EJ, Muslimov IA, Bianchi R, Reilly J, Weedon J, Diallo R, Bocker W, Tiedge H. BC200 RNA in invasive and preinvasive breast cancer. Carcinogenesis 2004;25:2125–2133.
15. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153–156.
16. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339–346.

17. Roskams T, De Vos R, Desmet V. "Undifferentiated progenitor cells" in focal nodular hyperplasia of the liver. Histopathology 1996;28:291–299.
18. Hegde P, Qi R, Abernathy K, Gay C, Dharap S, Gaspard R, Hughes JE, Snesrud E, Lee N, Quackenbush J. A concise guide to cDNA microarray analysis. BioTechniques 2000;29:548–550.
19. Hrzenjak A, Moinfar F, Tavassoli F, Strohmeier B, Kremser M-L, Zatloukal K, Denk H. JAZF1/JJAZ1 gene fusion in endometrial stroma sarcomas. J Mol Diagn 2005;7:388–395.
20. Fickert P, Trauner M, Fuchsbichler A, Stumptner C, Zatloukal K, Denk H. Cytokeratins as targets for bile acid-induced toxicity. Am J Pathol 2002;160:491–499.
21. Wang Z, Brown DD. A gene expression screen. Proc Natl Acad Sci U S A 1991;88:11505–11509.
22. Christa L, Simon MT, Flinois JP, Gebhardt R, Brechot C, Lasserre C. Overexpression of glutamine synthetase in human primary liver cancer. Gastroenterology 1994;106:1312–1320.
23. Santoni-Rugiu E, Jensen MR, Thorgeirsson SS. Disruption of the pRb/E2F pathway and inhibition of apoptosis are major oncogenic events in liver constitutively expressing c-myc and transforming growth factor alpha. Cancer Res 1998;58:123–134.
24. Smit A. Identification of a new, abundant superfamily of mammalian LTR-transposons. Nucleic Acids Res 1993;2:1863–1872.
25. Skryabin BV, Kremerskothen J, Vassilacopoulou D, Disotell TR, Kapitonov VV, Jurka J, Brosius J. The BC200 RNA gene and its neural expression are conserved in Anthropoidea (Primates). J Mol Evol 1998;47:677–685.
26. Mattick JS. Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. Bioessays 2003;25: 930–939.
27. Cui H, Onyango P, Brandenburg S, Wu Y, Hsieh CL, Feinberg AP. Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2. Cancer Res 2002;62:6442–6446.
28. Zalfa F, Giorgi M, Primerano B, Moro A, Di Penta A, Reis S, Oostra B, Bagni C. The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. Cell 2003;112:317–327.
29. Parmar KM, Larman B, Dai G, Zhang Y, Wang ET, Moorthy SN, Kratz JR, Lin Z, Jain MK, Gimbrone MA, Garcia-Cardena G. Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. J Clin Invest 2006;116:49–58.
30. Chen X, Cheung ST, So S, Fan ST, Barry C, Higgins J, Lai KM, Ji J, Dudoit S, Ng IO, Van De Rijn M, Botstein D, Brown PO. Gene expression patterns in human liver cancers. Mol Biol Cell 2002; 13:1929–1939.
31. Ye QH, Qin LX, Forgues M, He P, Kim JW, Peng AC, Simon R, Li Y, Robles AI, Chen Y, Ma ZC, Wu ZQ, Ye SL, Liu YK, Tang ZY, Wang XW. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 2003;9:416–423.
32. Xu XR, Huang J, Xu ZG, Qian BZ, Zhu ZD, Yan Q, Cai T, Zhang X, Xiao HS, Qu J, Liu F, Huang QH, Cheng ZH, Li NG, Du JJ, Hu W, Shen KT, Lu G, Fu G, Zhong M, Xu SH, Gu WY, Huang W, Zhao XT, Hu GX, Gu JR, Chen Z, Han ZG. Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding non-cancerous liver. Proc Natl Acad Sci U S A 2001;98:15089–15094.
33. Eferl R, Ricci R, Kenner L, Zenz R, David JP, Rath M, Wagner EF. Liver tumor development. c-Jun antagonizes the proapoptotic activity of p53. Cell 2003;112:181–192.
34. Feitelson MA, Sun B, Satiroglu Tufan NL, Liu J, Pan J, Lian Z. Genetic mechanisms of hepatocarcinogenesis. Oncogene 2002; 21:2593–2604.
35. Cadoret A, Ovejero C, Terris B, Souil E, Levy L, Lamers WH, Kitajewski J, Kahn A, Perret C. New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism. Oncogene 2002;21:8293–8301.
36. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–466.
37. Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001;61:2129–2137.
38. Brosius J. Gene duplication and other evolutionary strategies: from the RNA world to the future. J Struct Funct Genomics 2003; 3:1–17.
39. Brosius J, Gould SJ. On "genomenclature": a comprehensive (and respectful) taxonomy for pseudogenes and other "junk DNA." Proc Natl Acad Sci U S A 1992;89:10706–10710.
40. Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. Cell 2004;117:83–94.
41. de Kok JB, Verhaegh GW, Roelofs RW, Hessels D, Kiemeney LA, Aalders TW, Swinkels DW, Schalken JA. DD3(PCA3), a very sensitive and specific marker to detect prostate tumors. Cancer Res 2002;62:2695–2698.

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